1640 medium containing 10% FCS and either simian rIL-15 (Genzyme, 60 ng ml^{-1}) or murine recombinant IL-7 (R&D, 10 ng ml^{-1}) for 10 days. Cells recovered from cultures were analysed by flow cytometry or in the cytotoxicity assay.

Plasmid construction and luciferase assay. A DNA fragment spanning from positions −774 to +213 of the *IL-15* gene (N.A. and Y.T., unpublished observations) was cloned into a luciferase reporter plasmid (Promega) either directly or after deleting 5′ portions. Synthetic oligomers representing the potential IRF-E in the promoter region of the *IL-15* gene were inserted along with downstream human IFN-β-promoter elements (−55 to +19 region) into the Picagene luciferase-reporter plasmid (Wako)[30]. Reporter constructs were then transfected with IRF-1-expression (pAct-1) or control (pAct-C) vectors into P19 cells, which express no endogenous IRF-1, and luciferase activity was measured as previously described[30].

Analysis of *IL-15* gene expression. Bone marrow cells were cultured in the presence of LPS (30 μg ml^{-1}) and IFN-γ (100 units ml^{-1}). Total RNA was isolated by acid guanidium thiocyanate–phenol–chloroform extraction and subjected to northern blot analysis as previously described[30]. Mouse *IL-15* and β-actin complementary DNA probes obtained by PCR were used for hybridization.

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1. Trinchieri, G. Biology of natural killer cells. *Adv. Immunol.* **47**, 187–376 (1989).
2. Scott, P. & Trinchieri, G. The role of natural killer cells in host–parasite interactions. *Curr. Opin. Immunol.* **7**, 34–40 (1995).
3. Haller, O. & Wigzell, H. Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow dependent. *J. Immunol.* **118**, 1503–1506 (1977).
4. Seaman, W. E., Gindhart, T. D., Greenspan, J. S., Blackman, M. A. & Talal, N. Natural killer cells, bone, and the bone marrow: studies in estrogen-treated mice and in congenitally osteopetrotic (mi/mi) mice. *J. Immunol.* **122**, 2541–2547 (1979).
5. Kumar, V., Ben-Ezra, J., Bennett, M. & Sonnenfeld, G. Natural killer cells in mice treated with ^{89}strontium: normal target-binding cell numbers but inability to kill even after interferon administration. *J. Immunol.* **123**, 1832–1838 (1979).
6. Hackett, J. Jr, Bennett, M. & Kumar, V. Origin and differentiation of natural killer cells. I. Characteristics of a transplantable NK cell precursor. *J. Immunol.* **134**, 3731–3738 (1985).
7. Duncan, G. S., Mittrücker, H.-W., Kägi, D., Matsuyama, T. & Mak, T. W. The transcription factor interferon regulatory factor-1 is essential for natural killer cell function *in vivo*. *J. Exp. Med.* **184**, 2043–2048 (1996).
8. Taki, S. *et al.* Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity* **6**, 673–679 (1957).
9. Giri, J. G. *et al.* Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* **13**, 2822–2830 (1994).
10. Grabstein, K. H. *et al.* Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Science* **264**, 965–968 (1994).
11. Carson, W. E. *et al.* Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med.* **180**, 1395–1403 (1994).
12. Tagaya, Y., Bamford, R. N., DeFilippis, A. P. & Waldmann, T. A. IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. *Immunity* **4**, 329–336 (1996).
13. Matsuyama, T. *et al.* Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell* **75**, 83–97 (1993).
14. Tanaka, T. *et al.* Selective long-term elimination of natural killer cells *in vivo* by an anti-interleukin 2 receptor β chain monoclonal antibody in mice. *J. Exp. Med.* **178**, 1103–1107 (1993).
15. Rolink, A. *et al.* A subpopulation of B220$^+$ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J. Exp. Med.* **83**, 187–194 (1996).
16. DiSanto, J. P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl Acad. Sci. USA* **92**, 377–381 (1995).
17. Ohbo, K. *et al.* Modulation of hematopoiesis in mice with a truncated mutant of the interleukin-2 receptor γ chain. *Blood* **87**, 956–967 (1996).
18. Suzuki, H., Duncan, G. S., Takimoto, H. & Mak, T. W. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor β chain. *J. Exp. Med.* **185**, 499–505 (1997).
19. DiSanto, J. P. Shared receptors, distinct functions. *Curr. Biol.* **7**, R424–R426 (1997).
20. Mrozek, E., Anderson, P. & Caligiuri, M. A. Role of interleukin-15 in the development of human CD56$^+$ natural killer cells from CD34$^+$ hematopoietic progenitor cells. *Blood* **87**, 2632–2640 (1996).
21. Puzanov, I. J., Bennett, M. & Kumar, V. IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells. *J. Immunol.* **157**, 4282–4285 (1996).
22. Anderson, D. M. *et al.* Chromosomal assignment and genomic structure of IL-15. *Genomics* **25**, 701–706 (1995).
23. Tanaka, N., Kawakami, T. & Taniguchi, T. Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. *Mol. Cell. Biol.* **13**, 4531–4538 (1993).
24. Bamford, R. N., Battiata, A. P., Burton, J. D., Sharma, H. & Waldmann, T. A. Interleukin (IL) 15/IL-T production by the adult T-cell leukemia cell line HuT-102 is associated with a human T-cell lymphotrophic virus type I region/IL-15 fusion message that lacks many upstream AUGs that normally attenuates IL-15 mRNA translation. *Proc. Natl Acad. Sci. USA* **93**, 2897–2902 (1996).
25. Taniguchi, T., Lamphier, M. S. & Tanaka, N. IRF-1: the transcription factor linking the interferon response and oncogenesis. *Biochim. Biophys. Acta* **1333**, M9–M17 (1997).
26. Dalton, D. K. *et al.* Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science* **259**, 1739–1742 (1993).
27. Huang, S. *et al.* Immune response in mice that lack the interferon-γ receptor. *Science* **259**, 1742–1745 (1993).
28. Bendelac, A., Rivera, M. N., Park, S. H. & Roark, J. H. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* **15**, 535–562 (1997).
29. Rodewald, H. R. *et al.* A population of early fetal thymocytes expressing FcγRII/III contains precursors of T lymphocytes and natural killer cells. *Cell* **69**, 139–150 (1992).
30. Tan, R. S., Taniguchi, T. & Harada, H. Identification of the lysyl oxidase gene as target of the antioncogenic transcription factor, IRF-1, and its possible role in tumor supression. *Cancer Res.* **56**, 2417–2421 (1996).

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Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells

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Natural killer (NK) cells express cell-surface receptors of the immunoglobulin and C-type lectin superfamilies that recognize major histocompatibility complex (MHC) class I peptides and inhibit NK-cell-mediated cytotoxicity[1]. These inhibitory receptors possess ITIM sequences (for immunoreceptor tyrosine-based inhibitory motifs) in their cytoplasmic domains that recruit SH2-domain-containing protein tyrosine phosphatases, resulting in inactivation of NK cells[2–4]. Certain isoforms of these NK-cell receptors lack ITIM sequences and it has been proposed that these 'non-inhibitory' receptors may activate, rather than inhibit, NK cells[4–6]. Here we show that DAP12, a disulphide-bonded homodimer containing an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain, non-covalently associates with membrane glycoproteins of the killer-cell inhibitory receptor (KIR) family without an ITIM in their cytoplasmic domain. Crosslinking of KIR–DAP12 complexes results in cellular activation, as demonstrated by tyrosine phosphorylation of cellular proteins and upregulation of early-activation antigens. Phosphorylated DAP12 peptides bind ZAP-70 and Syk protein tyrosine kinases, suggesting that the activation pathway is similar to that of the T- and B-cell antigen receptors.

It has been reported that an unknown phosphoprotein of relative molecular mass (M_r) ~12,000, expressed as a disulphide-bonded dimer, was coimmunoprecipitated from NK-cell lysates together with a non-inhibitory KIR2DS2 glycoprotein (a KIR family member with two immunoglobulin-domains in the extracellular domain, a short cytoplasmic domain lacking an ITIM, and a charged residue in the transmembrane region that is a receptor for HLA-C ligands, also referred to as p50.2 or KAR[7]). Cell-surface immunoglobulin receptors, T-cell antigen receptors (TCR), and certain Fc receptors (FcR) non-covalently associate with small transmembrane proteins (such as CD3δ,γ,ε,ζ subunits, CD79α,β, FcεRI-γ) containing ITAM sequences (D/ExxYxxL/I − x_{6-8} − YxxL/I)[8] that are required for signal transduction by these receptor complexes[9]. Therefore, it seems likely that these non-inhibitory NK-cell receptors might require an associated protein with similar properties to mediate positive signal transduction.

A database of expressed tag sequences (EST) from a large panel of complementary DNA libraries was searched with a TBLASTN algorithm program for molecules bearing homology with the human CD3δ,γ,ε,ζ and FcεRI-γ protein sequences. An EST from a human CD1+ dendritic cell library was selected for further study

because of identification of an ITAM in this molecule. Sequencing of the 604 base pair (bp) cDNA revealed an open reading frame of 339 nucleotides, encoding a putative type I membrane protein of 113 amino acids (Fig. 1a). The protein, designated DAP12, is composed of a 27 amino-acid leader, 14 amino-acid extracellular domain, 24 amino-acid transmembrane segment and 48 amino-acid cytoplasmic region. Although DAP12 has less than 25% homology with the human CD3δ,γ,ε,ζ and FcεRI-γ proteins, the cytoplasmic domain contains the peptide ESPYQELQGQRSDVYSDL that precisely corresponds to the prototype ITAM consensus (Fig. 1b). Potential sites for phosphorylation by protein kinase C (residues 79–81 and 107–109) and casein kinase II (residues 85–88) are also present in the DAP12 cytoplasmic region. The transmembrane region contains a charged amino acid (aspartic acid (D)), also conserved in the transmembrane domain of the CD3 subunits. A potential murine homologue of DAP12 is ~70% homologous with the human DAP12 protein and has a conserved D residue in the transmembrane region, conserved cysteine (C) residues in the extracellular domain and an ITAM in the cytoplasmic region (Fig. 1c).

Southern blot analysis of human genomic DNA revealed a restriction enzyme digest pattern that is consistent with a single *DAP12* gene (Fig. 2a). *DAP12* is located on human chromosome 19q13.1. The human *KIR* genes[10] and the related *LAIR*[11] and *ILT/MIR*[12] genes are all located nearby on chromosome 19q13.4. Northern blot analysis indicated the abundant presence of ~0.7 kilobase (kb) DAP12 transcripts in human peripheral blood leukocytes and spleen, but not in thymus, prostate, testis, ovary, small intestine or colon (Fig. 2b). DAP12 transcripts were detected in RNA isolated from two human NK cell lines NKL and NK92, but not in the Jurkat T leukaemia cell line or the JY EBV-transformed B lymphoblastoid cell line (Fig. 2b). Southern blot analysis of a large panel of cDNA libraries revealed predominant expression of DAP12 in resting human peripheral blood mononuclear cells, dendritic cells (from which DAP12 was cloned), peripheral blood monocytes and NK cell lines and clones (not shown).

A conspicuous feature of the non-inhibitory KIR[5], Ly49D[6], Ly49H[6], CD94[13], NKG2C[14] and ILT1[15] receptors is the presence of a basic amino acid (K (lysine) or R (arginine)) in the transmembrane domain. Given the precedent for interactions between proteins of multisubunit receptor complexes through oppositely charged amino acids in the transmembrane domains (such as the CD3/TCR complex)[9], we examined whether DAP12 associates with the non-inhibitory KIR2DS2 glycoprotein containing a K in the transmembrane region[16]. The murine Ba/F3 pre-B cell line was transfected with a cDNA encoding KIR2DS2 either alone or together with a DAP12 cDNA containing a FLAG epitope tag at the N terminus to permit detection with an anti-FLAG monoclonal antibody. Transfectants were selected by flow cytometry for cell-surface expression based on a positive staining result with anti-KIR antibody DX27 or anti-FLAG antibody M2. KIR2DS2 Ba/F3 and KIR2DS2 + DAP12–FLAG Ba/F3 transfectants were surface-labelled with ^{125}I, lysed in 1% digitonin to preserve non-covalent associations of membrane protein complexes, and immunoprecipitated with anti-KIR antibody or anti-FLAG antibody. The tyrosine residue in the FLAG epitope provided a site for radioiodination, permitting visualization of the DAP12 protein. As shown in Fig. 3a, anti-KIR antibody immunoprecipitated a ^{125}I-labelled species of ~50–60K from both the KIR2DS2 Ba/F3 cells and KIR2DS2 + DAP12–FLAG Ba/F3 transfectants, which is consistent with the predicted molecular mass of the KIR2DS2 glycoprotein. An additional ^{125}I-labelled protein of ~12K was coimmunoprecipitated with anti-KIR antibody from the

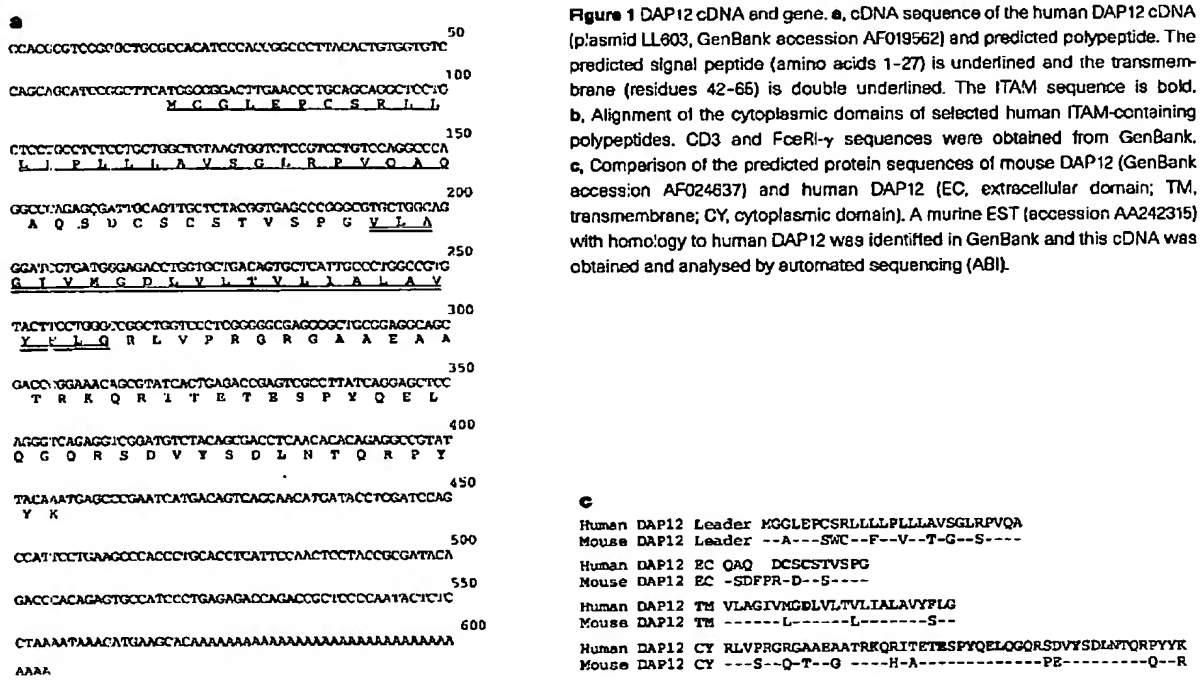

Figure 1 DAP12 cDNA and gene. **a**, cDNA sequence of the human DAP12 cDNA (plasmid LL603, GenBank accession AF019562) and predicted polypeptide. The predicted signal peptide (amino acids 1–27) is underlined and the transmembrane (residues 42–65) is double underlined. The ITAM sequence is bold. **b**, Alignment of the cytoplasmic domains of selected human ITAM-containing polypeptides. CD3 and FcεRI-γ sequences were obtained from GenBank. **c**, Comparison of the predicted protein sequences of mouse DAP12 (GenBank accession AF024637) and human DAP12 (EC, extracellular domain; TM, transmembrane; CY, cytoplasmic domain). A murine EST (accession AA242315) with homology to human DAP12 was identified in GenBank and this cDNA was obtained and analysed by automated sequencing (ABI).

KIR2DS2 + DAP12–FLAG transfectant, but not from the transfectant expressing only KIR2DS2. Reciprocally, an ^{125}I-labelled glycoprotein migrating in an identical way to KIR2DS2 was coimmunoprecipitated with anti-FLAG antibody from the KIR2DS2 + DAP12–FLAG Ba/F3 cells, but not from the KIR2DS2-only transfectant. Comparison of immunoprecipitates analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using either reducing or non-reducing conditions indicate that DAP12 is expressed on the cell surface as a disulphide-bonded dimer (Fig. 3a). It should be noted that we were unable to detect cell-surface expression of DAP12 on the surface of Ba/F3 cells transfected with the DAP12–FLAG cDNA alone, without KIR2DS2. However DAP12–FLAG proteins were detected in the cytoplasm (not shown), suggesting that DAP12 may require association with its partner subunits for efficient transport to the cell surface, in a similar manner to the situation with the CD3 proteins[17]. Additionally, preliminary results indicate that DAP12 does not associate with the inhibitory KIR isoforms that lack a charged residue in their transmembrane domain (unpublished observation).

A peptide corresponding to the cytoplasmic domain of DAP12 (ITETESPY*QELQGQRSDVY*SDLNTQRP) was synthesized either as an unphosphorylated protein or containing phosphates on both tyrosine (Y) residues. Lysates from Jurkat T cells or NK cell clone A6 were incubated with the biotinylated peptides and complexes precipitated using avidin–agarose. Western blot analysis demonstrated that a DAP12 peptide phosphorylated on both Y residues, but not the unphosphorylated peptide, formed complexes with the ZAP-70 kinase (Fig. 3b). The tyrosine-phosphorylated DAP12 peptide, but not the unphosphorylated DAP12 peptide, also formed a complex with the Syk protein tyrosine kinase in lysates from NK cells (not shown). The binding of these kinases to phosphorylated DAP12 is reminiscent of the interactions that have been demonstrated between the phosphorylated ITAM-containing CD3 subunits and Syk or ZAP-70 kinases during TCR signalling[18,19].

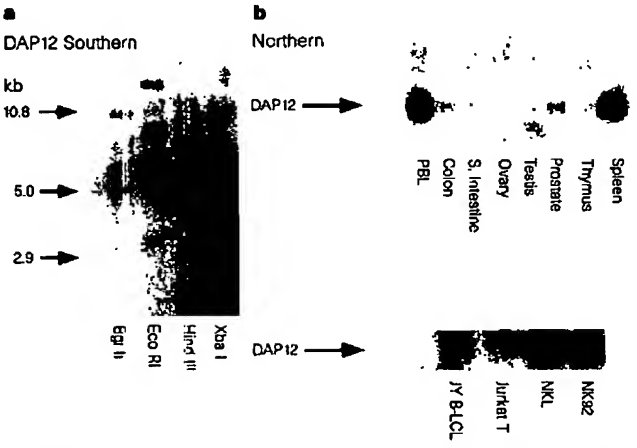

Figure 2 DAP12 gene and expression. **a**, Southern blot analysis of human genomic DNA digested with the indicated restriction enzymes and probed with the DAP12 cDNA. The restriction enzyme fragments are in accordance with the sizes predicted from the genomic sequence. The genomic organization of the human DAP12 gene (GenBank accession AF019563) was deduced from a fragment of human chromosome 19q13.1 (GenBank accession AD000833). **b**, Northern blot analysis of DAP12 in human tissues and the JY EBV-transformed B lymphoblastoid cell line, the Jurkat T leukaemia cell line, and two NK cell lines NKL (provided by M. Robertson, Indiana University) and NK92 (obtained from H-G. Klingemann).

Ligation of the CD3/TCR complex on T cells or the immunoglobulin receptor complex on B cells results in cellular activation. Therefore, we examined the functional consequence of crosslinking the KIR2DS2–DAP12 complex. Ba/F3 transfectants expressing either KIR2DS2 alone or the KIR2DS2–DAP12–FLAG complex were incubated with anti-KIR antibody DX27 or anti-FLAG antibody, followed by a goat anti-mouse immunoglobulin to provide crosslinking. Examination of total cellular proteins in Ba/F3 cells expressing the KIR2DS2–DAP12–FLAG complex that were stimulated with anti-KIR or anti-FLAG antibodies revealed tyrosine phosphorylation of several cellular substrates (Fig. 4a). Immunoprecipitation with anti-FLAG antibody and western blot analysis with anti-phosphotyrosine antibody demonstrated that crosslinking

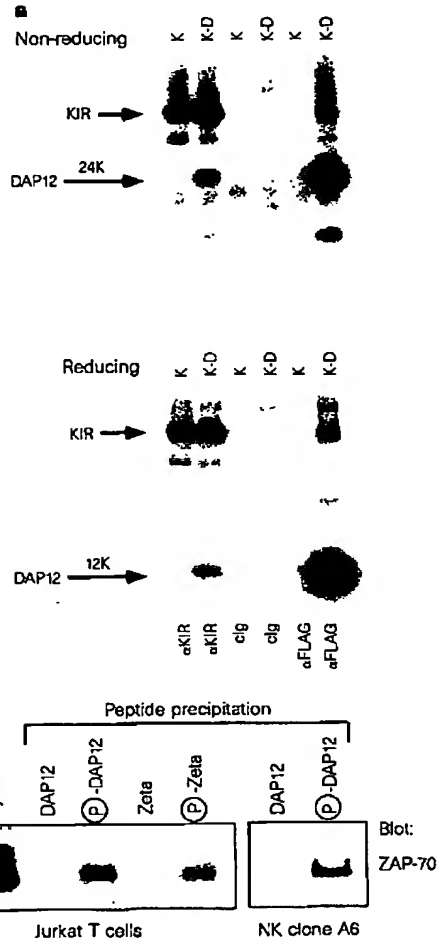

Figure 3 Coimmunoprecipitation of DAP12 and KIR2DS2 proteins and association of DAP12 phosphopeptide with ZAP-70. **a**, Murine Ba/F3 pre-B cells stably transfected with KIR2DS2 only (K) or KIR2DS2 and DAP12 (containing a FLAG epitope on the N terminus) (K-D) were labelled with ^{125}I, lysed in 1% digitonin buffer and antigens immunoprecipitated with control immunoglobulin (cig), anti-KIR antibody DX27 or anti-FLAG antibody M2. Samples were analysed by SDS-PAGE using 18% acrylamide gels under non-reducing or reducing conditions. **b**, Lysates prepared from Jurkat cells or NK cell clone A6 were incubated with a biotinylated unphosphorylated (DAP12) or diphosphorylated (P-DAP12) DAP12 peptide (ITETESPY*QELQGQRSDVY*SDLNTQRP) and precipitated with avidin-agarose. Samples were analysed by western blot using anti-ZAP-70 antibody, as indicated. Biotinylated unphosphorylated or diphosphorylated CD3ζ (P-zeta) peptides were used as a control in the experiment using lysates from Jurkat cells[8].

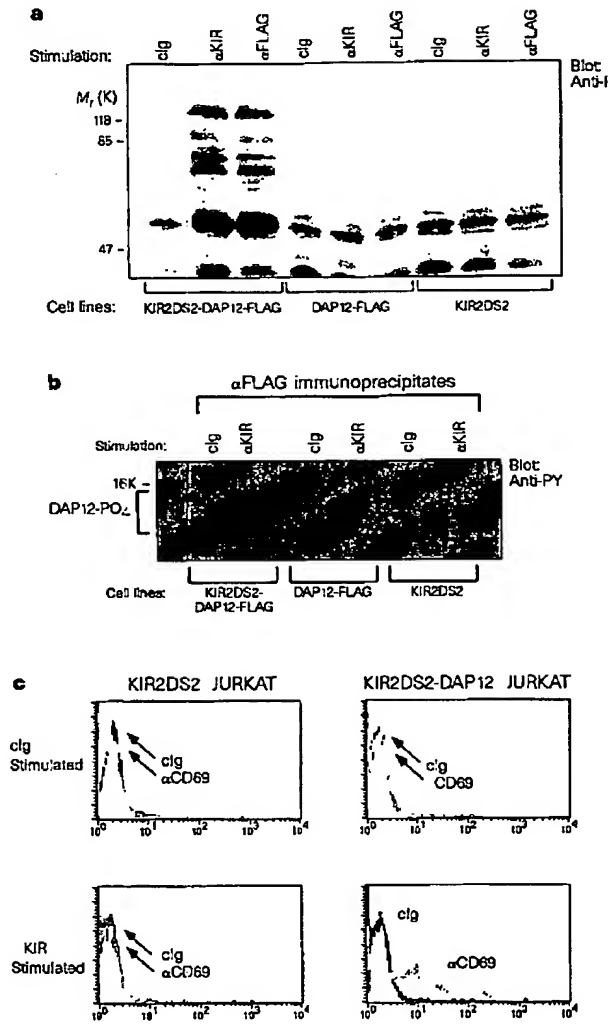

Figure 4 Cellular activation of transfectants expressing KIR2DS2 and DAP12. **a**, Total cell lysates were prepared from Ba/F3 cells transfected with KIR2DS2 alone, DAP12 (containing a FLAG epitope tag) alone or both KIR2DS2 and DAP12-FLAG that were stimulated by anti-KIR antibody DX27, anti-FLAG antibody M2 or control immunoglobulin, followed by F(ab')$_2$ goat anti-mouse immunoglobulin. Samples were analysed by western blot for the presence of phosphorylated cellular proteins using HRP-conjugated anti-phosphotyrosine antibody 4G10. **b**, Cell lysates were prepared from Ba/F3 cells transfected with KIR2DS2 alone or both KIR2DS2and DAP12 (FLAG epitope tagged) that were stimulated with anti-KIR antibody DX27 or control immunoglobulin, followed by F(ab')$_2$ goat anti-mouse immunoglobulin. Lysates were immunoprecipitated with anti-FLAG antibody M2 or control immunoglobulin and samples were analysed by western blot for the presence of phosphorylated proteins using HRP-conjugated anti-phosphotyrosine antibody 4G10. Heterogeneity in migration of the DAP12-FLAG proteins probably reflects different phosphorylation species. **c**, Jurkat cells were stably transduced with an amphotrophic retroviral vector pMX-neo[24] containing the NKAT5 cDNA[16] encoding KIR2DS2. These KIR2DS2+ Jurkat cells were transiently transfected by electroporation with human DAP12 in the pEF-BOS vector[19]. After 24 h, sham (KIR2DS2 Jurkat) or DAP12 transfectants (KIR2DS2-DAP12 Jurkat) were stimulated by culture in microtitre plates precoated (5 μg ml^{-1}) with control immunoglobulin (upper panels) or anti-KIR antibody DX27 (lower panels). After 12 h incubation, cells were collected and stained with FITC-conjugated control immunoglobulin (cIg) or anti-CD69 antibody, as indicated. Samples were analysed by flow cytometry (x-axis, fluorescence, 4 decade log scale; y-axis, number of cells).

the KIR2DS2–DAP12–FLAG transfectants with anti-KIR antibody induced tyrosine phosphorylation of the DAP12 protein (Fig. 4b) and resulted in the association of phosphorylated DAP12 with the Syk protein tyrosine kinase (not shown). By contrast, Ba/F3 cells expressing only KIR2DS2 were not activated by crosslinking with anti-KIR antibody. Similarly, upregulation of CD69 expression was observed in Jurkat T leukaemia cells transfected with both KIR2DS2 and DAP12, but not KIR2DS2 alone, when these receptors were crosslinked with anti-KIR antibody (Fig. 4c). These results indicate that DAP12 is necessary and responsible for KIR2DS2 signal transduction in these host cells and are in accordance with previous observations demonstrating that KIR2DS2 molecules are functional in NK cells but not in transfectants expressing only KIR2DS2[20].

Our studies suggest that DAP12 may associate with the non-inhibitory isoforms of the KIR molecular in NK cells and permit cellular activation through these receptors, in a similar way to the function of the CD3 subunits in the TCR complex and CD79 subunits in the B-cell-receptor complex. Whether DAP12 associates with the non-inhibitory NK-cell receptors of the murine Ly49 family and the human CD94-NKG2C and NKG2E complexes is under investigation. Expression of DAP12 in monocytes and dendritic cells predicts association with other receptors similar to the non-inhibitory KIR present in these cell types. Likely candidates are the recently identified ILT/MIR family of molecules expressed by human monocytes[12,15] and the PIR-A molecules in rodent myeloid and B cells[21,22]. In addition, the physical properties of DAP12 are similar to a new dimeric 12K phosphoprotein identified in the pre-T-cell-receptor complex on murine thymocytes[23]. Thus, DAP12 may function in cellular activation mediated by a diverse array of receptors in distinct cell lineages. □

Methods

Cloning and sequence analysis. TBLASTN searches of the DNAX sequence database were made using the human CD3δ,γ,ε,ζ and FcεRI-γ protein sequences. The cDNA insert in plasmid LL603, identified in a human CD1+ dendritic cell library, was isolated and subjected to automated sequencing (ABI).

DNA and RNA. RNA from human tissues and human genomic DNA were purchased from Clontech (Palo Alto, CA). Northern and Southern blot analysis were done as described[13].

Transfection. A cDNA containing the CD8 leader segment, followed by the FLAG peptide epitope (DYKDDDDK), and joined to the extracellular, transmembrane and cytoplasmic segments of DAP12 was subcloned into the pMX-puro retroviral vector[24] (provided by T. Kitamura, DNAX), packaged using the Phoenix cell line (provided by G. Nolan, Stanford), and virus was used to infect the mouse pre-B cell line Ba/F3[24]. The NKAT5 cDNA[16] encoding KIR2DS2 (provided by M. Colonna, Basel) was subcloned into the pMX-neo retroviral vector. Ba/F3 cells were infected, drug selected, and transfectants isolated using flow cytometry[24]. DAP12 cDNA was subcloned into the pEF-BOS vector for transient expression in Jurkat cells using electroporation for introduction of the plasmid[25].

Immunoprecipitation. Cells were labelled with ^{125}I and solubilized in lysis buffer (pH 7.8, 1% digitonin (Sigma), 0.12% Triton-X100, 150 mM NaCl, 20 mM triethanolamine, 0.01% NaN_3, and protease inhibitors)[26]. Cell lysates were incubated on ice for 2 h with Pansorbin (Calbiochem) coated with rabbit anti-mouse immunoglobulin (Sigma) and mouse anti-KIR2D antibody DX27, anti-FLAG antibody M2 (Kodak), or control IgG and then washed in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 8.0) containing 5 mM CHAPS (Sigma) and protease inhibitors[26]. Biotinylated peptides corresponding to residues ITETESPY*QELQGQRSDVY*SDLNTQRP in the cytoplasmic domain of DAP12 were synthesized, either unphosphorylated or containing phosphate on both Y residues (provided by C. Turck, UCSF). Control unphosphorylated and Y-phosphorylated CD3ζ peptides[19] were a gift from A. Weiss (UCSF). Biotinylated peptides were incubated with lysates from Jurkat or NK clone A6 cells, precipitated with avidin–agarose, and washed in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.8) containing 1% Nonidet P-40 and protease inhibitors[19]. Immunoprecipitates were analysed by western blot[27]

using anti-ZAP-70 antibody or rabbit anti-Syk specific antiserum[19] (provided by A. Weiss, UCSF).

Cell activation. Ba/F3 cells expressing either KIR2DS2 alone, DAP12 (FLAG epitope tagged) alone, or the KIR2DS2–DAP12 complex were incubated with the indicated antibodies at 4 °C, washed, and then crosslinked with $F(ab')_2$ goat anti-mouse immunoglobulin for 3 min at 37 °C. Cells were lysed in TBS containing 1% Nonidet P-40 and protease inhibitors. Total cell lysates or immunoprecipitates of DAP12–FLAG with anti-FLAG antibody M2 were analysed by western blot using horseradish peroxidase-conjugated anti-phosphotyrosine antibody 4G10 (UBI). Jurkat cells stably transfected with the NKAT5 cDNA[16] using a retroviral vector[24] were transiently transfected by electroporation with human DAP12 cDNA in the pEF-BOS vector or sham-transfected with a control vector[25]. After 24 h, transfectants were incubated in microtitre plates precoated (5 $\mu g\,ml^{-1}$) with control immunoglobulin or anti-KIR antibody DX27. After 12 h incubation, transfectants were collected and then stained with FITC-conjugated anti-CD69 or control antibody and analysed by flow cytometry[26].

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1. Lanier, L. L. NK cells: from no receptors to too many. *Immunity* 6, 371–378 (1997).
2. Burshtyn, D. N. *et al.* Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor. *Immunity* 4, 77–85 (1996).
3. Olcese, L. *et al.* Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J. Immunol.* 156, 4531–4534 (1996)
4. Houchins, J. P., Lanier, L. L., Niemi, E., Phillips, J. H. & Ryan, J. C. Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C. *J. Immunol.* 158, 3603–3609 (1997).
5. Biassoni, R. *et al.* The human leukocyte antigen (HLA)-C-specific 'activatory' or 'inhibitory' natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J. Exp. Med.* 183, 645–650 (1996).
6. Mason, L. H. *et al.* The Ly-49D receptor activates murine natural killer cells. *J. Exp. Med.* 184, 2119–2128 (1996).
7. Olcese, L. *et al.* Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by Natural Killer cells. *J. Immunol.* 158, 5083–5086 (1997).
8. Reth, M. Antigen receptor tail clue. *Nature* 338, 383–384 (1989).
9. Chan, A.C., Desai, D. M. & Weiss, A. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu. Rev. Immunol.* 12, 555–592 (1994).
10. Baker, E., D'Andrea, A., Phillips, J. H., Sutherland, G. R. & Lanier, L. L. Natural killer cell receptor for HLA-B allotypes. NKB1: map position 19q13.4. *Chrom. Res.* 3, 511 (1995).
11. Meyaard, L. *et al.* LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity* 7, 283–290 (1997).
12. Wagtmann, N., Rojo, S., Eichler, E., Mohrenweiser, H. & Long, E. O. A new human gene complex encodes the killer cell inhibitory receptors and a related family of monocyte/macrophage receptors. *Curr. Biol.* 7, 615–618 (1997).
13. Chang, C. *et al.* Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. *Eur. J. Immunol.* 25, 2433–2437 (1995).
14. Houchins, J. P., Yabe, T., McSherry, C. & Bach, F. H. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J. Exp. Med.* 173, 1017–1020 (1991).
15. Samaridis, J. & Colonna, M. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur. J. Immunol.* 27, 660–665 (1997).
16. Colonna, M. & Samaridis, J. Cloning of Ig-superfamily members associated with HLA-C and HLA-B recognition by human NK cells. *Science* 268, 405–408 (1995).
17. Clevers, H., Alarcon, B., Wileman, T. & Terhorst, C. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6, 629–662 (1988).
18. Iwashima, M., Irving, B. A., van Oers, N. S. C., Chang, A. C. & Weiss, A. Sequential Interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* 263, 1136–1139 (1994).
19. Chan, A. C. *et al.* Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TcR signaling. *J. Immunol.* 152, 4758–4766 (1994).
20. Blery, M. *et al.* Reconstituted killer cell inhibitory receptors for major histocompatibility complex class I molecules control mast cell activation induced via immunoreceptor tyrosine-based activation motifs. *J. Biol. Chem.* 272, 8989–8996 (1997).
21. Hayami, K. *et al.* Molecular cloning of a novel murine cell-surface glycoprotein homologous to killer cell inhibitory receptors. *J. Biol. Chem.* 272, 7320–7326 (1997).
22. Kubagawa, H., Burrows, P. D. & Cooper, M. D. A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells. *Proc. Natl Acad. Sci. USA* 94, 5261–5266 (1997).
23. Takase, K. *et al.* A new 12-kilodalton dimer associated with pre-TCR complex and clonotype-independent CD3 complex on immature thymocytes. *J. Immunol.* 159, 741–747 (1997).
24. Onishi, M. *et al.* Applications of retrovirus-mediated expression cloning. *Exp. Hematol.* 24, 324–329 (1996).
25. Wu, J., Katzav, S. & Weiss, A. A functional T-cell receptor signaling pathway is required for p95vav activity. *Mol. Cell. Biol.* 15, 4337–4346 (1995).
26. Lanier, L. L., Yu, G. & Phillips, J. H. Co-association of CD3ζ with a receptor (CD16) for IgG Fc on human natural killer cells. *Nature* 342, 803–805 (1989).
27. Phillips, J. H. *et al.* CD94 and a novel associated protein (94AP) form a NK cell receptor involved in the recognition of HLA-A, -B, and -C allotypes. *Immunity* 5, 163–172 (1996).
28. Lanier, L. L. & Recktenwald, D. J. Multicolor immunofluorescence and flow cytometry. *Methods: A Companion to Methods in Enzymology* 2, 192–199 (1991).

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Involvement of p85 in p53-dependent apoptotic response to oxidative stress

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Reactive oxygen species have damaging effects on cellular components and so trigger defensive responses by the cell[1,2] and even programmed cell death[3,4], although the mechanisms by which mammalian cells transmit signals in response to oxidative damage are unknown. We report here that the protein p85, a regulator of the signalling protein phosphatidyl-3-OH kinase (PI(3)K), participates in the cell death process that is induced in response to oxidative stress and that this role of p85 in apoptosis does not involve PI(3)K. We show that disruption of p85 by homologous recombination impairs the cellular apoptotic response to oxidative stress. Because the protein p53 is required for cell death induced by oxidative damage, we examined the relation between p85 and p53. Using a chimaeric p53 fusion protein with the oestrogen receptor (p53ER) to supply p53 (p53 is induced upon binding of p53ER to oestradiol) in a p53-deficient cell line, we found that p85 is upregulated by p53 and that its involvement in p53-mediated apoptosis is independent of PI(3)K. We propose that p85 acts as a signal transducer in the cellular response to oxidative stress, mediating cell death regulated by p53.

We reasoned that cellular responses to oxidative stress might be mediated by signal-transducing proteins. Hydrogen peroxide is required to mediate signal transduction by the receptor for platelet-derived growth factor[5], which associates with p85 protein[6]. p85 is an SH2/SH3-domain-containing protein originally identified as a regulator of phosphatidylinositol-3-OH kinase[7] and possibly of other signal transduction proteins[8]. To study the role of p85 in the cellular response to oxidative stress, we derived primary mouse embryo fibroblasts (MEFs) from mice with a targeted disruption of the *p85* alpha gene locus (Y.T. *et al.*, manuscript in preparation). $p85^{+/+}$ MEFs from wild-type mice with an otherwise identical genetic background were used for comparison. As expected, expression of p85 was normal in $p85^{+/+}$ MEFs but was undetectable in $p85^{-/-}$ MEFs (Fig. 1a). The amount of p85 in normal MEFs increased following treatment with 88 μM H_2O_2 (Fig. 1b; compare lanes 3 and 4). However, the concentration of p85 was unaffected by H_2O_2 in p53-deficient MEFs (Fig. 1b; compare lanes 5 and 6), suggesting that there might be a link between p53 function and the response of p85 to oxidative stress. To determine the effect of disrupting *p85* on the cell death pathway, $p85^{+/+}$ and $p85^{-/-}$ MEF cells were exposed to H_2O_2. As shown in Fig. 1c, $p85^{+/+}$ cells were susceptible to H_2O_2 treatment, with more than 60% of $85^{+/+}$ cells being killed after 24 hours' exposure to 88 μM H_2O_2. In contrast, $p85^{-/-}$ MEF cells were resistant to killing by the same dose of H_2O_2

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